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Practitioner's Docket No. U 012121-2 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Maarten Hendrik STUIVER, et al
Serial No.: 09/258,031 Group No.: 1652
Filed: February 25, 1999 Examiner: Richard G. Hutson
For: ANTIFUNGAL PROTEINS, DNA CODING THEREFORE, AND HOSTS INCORPORATING SAME

Commissioner for Patents
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Country:	The Netherlands
Application Number:	96202466.7
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Application Number:	97200831.2
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Patentanmeldung Nr. Patent application No. Demande de brevet n°

96202466.7

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Antifungal proteins, DNA coding therefor, and hosts incorporating same

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Antifungal proteins, DNA coding therefor, and hosts incorporating same

FIELD OF THE INVENTION

The present invention relates to antifungal proteins, DNA coding therefor and hosts incorporating the DNA, as well as methods of combating fungal pathogens by causing said fungal pathogens to be contacted with said protein or proteins.

The invention further relates to plants, incorporating and expressing DNA coding for antifungal proteins, and to plants which as a result thereof show reduced susceptibility to fungal pathogens, in particular to the Oomycetes *Phytophthora* and *Pythium*.

BACKGROUND ART

Phytophthora infestans belongs to the group of fungi referred to as Oomycetes. *Phytophthora infestans* infects various members of Solanaceae, such as potato, tomato and some ornamentals. It causes late blight of potatoes and tomatoes affecting all parts except roots. Geographically, the fungus is widely distributed, and it can be found in all potato-producing countries. Economically late blight in potatoes is of major importance, as infection early in the season can severely reduce crop yield. Currently the disease is controlled by spraying chemical fungicides (dithiocarbamates, such as mancozeb, manec and zineb) regularly. Both from an environmental and economical point of view, biological control of diseases caused by *Phytophthora infestans* could have advantages over the use of chemical fungicides.

Pythium also belongs to the group of fungi referred to as Oomycetes. The genus *Pythium* differs from the related genus *Phytophthora* by forming relatively undifferentiated sporangia. Geographically, this fungus is widely distributed on all continents. The first main type of disease caused by *Pythium* species is damping-off, due to sudden and fast developing attacks on young seedlings in the field or in nurseries. *Pythium* species cause a second type of disease which is root necrosis and causes a general slowing of plant growth (for example wheat and maize) and loss of yield.

The main losses caused by *Pythium* in Europe are to field crops such as sugarbeet. In principle, losses tend to be all-or-nothing. Similarly, nursery sowings of ornamentals and forest trees may be completely destroyed. (For a review on Oomycetes, *vide*: European Handbook of Plant Diseases, ed. by I.M. Smith *et al.*, 1988, Blackwell Scientific Publications, Ch.8)

A protein with antifungal activity, isolated from TMV-induced tobacco leaves, which is capable of causing lysis of germinating spores and hyphal tips of *Phytophthora infestans* and which causes the hyphae to grow at a reduced rate, was disclosed in WO91/18984 A1. This protein has an apparent molecular weight of about 24 kDa and was named AP24. Comparison of its complete amino acid sequence, as deduced from the nucleic acid sequence of the AP24 gene, with proteins known from databases revealed that the protein was an osmotin-like protein.

Despite initial success in combating fungal pathogens, such as *Phytophthora*

infestans, and the genetic engineering of plants capable of producing these antifungal proteins with activity against this fungal pathogen there remains a need to identify and isolate other proteins with antifungal activity against this fungus.

SUMMARY OF THE INVENTION

The present invention provides an isolated protein obtainable from a plant source which has anti- fungal activity, especially directed to Oomycetes, and preferably to *Phytophthora* and/or *Pythium* and a molecular weight of about 59 ± 5 kDa as judged by SDS PAGE-electrophoresis. A more preferred protein is one that is obtainable from sunflower plants. An even more preferred protein is one that is obtainable from sunflower leaves induced with sodium salicylate. A still more preferred isolated protein is characterised in that it is selected from the group of proteins having the amino acid sequence selected from the group comprising of the amino acid sequences depicted in SEQ ID NO's: 1, 2, 6, 11, 13 and 15, as well as muteins thereof which have antifungal and especially anti-*Phytophthora* and/or anti-*Pythium* activity. A still further preferred protein according to the invention is one characterised in that it comprises a protein that is being encoded by the nucleotides from the open reading frame represented by SEQIDNO: 14, or by a part of said open reading frame like represented in SEQ ID NO's: 5, 10 or 12.

The invention also embraces an isolated DNA sequence comprising an open reading frame capable of encoding a protein according to the invention, preferably characterised in that the open reading frame is capable of encoding a protein according to the invention, and DNA capable of hybridising therewith under stringent conditions.

The invention also provides a chimeric DNA sequence according to the invention further comprising a transcriptional initiation region and, optionally, a transcriptional termination region, so linked to said open reading frame as to enable the DNA to be transcribed in a living host cell when present therein, thereby producing RNA which comprises said open reading frame. A preferred chimeric DNA sequence according to the invention is one, wherein the RNA comprising said open reading frame is capable of being translated into protein in said host cell, when present therein, thereby producing said protein.

The invention also embraces a chimeric DNA sequence comprising a DNA sequence according to the invention, which may be selected from replicons, such as bacterial cloning plasmids and vectors, such as a bacterial expression vector, a (non-integrative) plant viral vector, a Ti-plasmid vector of *Agrobacterium*, such as a binary vector, and the like, as well as a host cell comprising a replicon or vector according to the invention, and which is capable of maintaining said replicon once present therein. Preferred according to that embodiment is a host cell which is a plant cell, said vector being a non-integrative viral vector.

The invention further provides a host cell stably incorporating in its genome a chimeric DNA sequence according to the invention, such as a plant cell, as well as multicellular hosts comprising such cells, or essentially consisting of such cells, such as plants. Especially preferred are plants characterised in that the chimeric DNA according to the invention is expressed in at least a

number of the plant's cells causing the said antifungal protein to be produced therein.

According to yet another embodiment of the invention a method for producing a protein with antifungal activity is provided, characterised in that a host cell according to the invention is grown under conditions allowing the said protein to be produced by said host cell, optionally followed by the step of recovering the protein from the host cells.

The invention provides also for the use of a protein according to the invention for retarding the growth of fungi, preferably Oomycetes and more preferably *Phytophthora* and *Pythium*, preferably characterised in that spores of the said fungus are caused to be contacted with said protein. According to yet another embodiment, retarding the growth of the fungus *Phytophthora* and/or *Pythium* is on plant leaves, characterised in that hyphae thereof, or spores thereof, are caused to be contacted with a protein produced from a host cell according to the invention capable thereof.

The invention also provides a method for obtaining plants with reduced susceptibility to fungi, especially *Phytophthora* and/or *Pythium*, comprising the steps of

(a) introducing into ancestor cells which are susceptible of regeneration into a whole plant,

- a chimeric DNA sequence comprising an open reading frame capable of encoding a protein according to claim 1, said open reading frame being operatively linked to a transcriptional and translational region and, optionally, a transcriptional termination region, allowing the said protein to be produced in a plant cell that is susceptible to infection by said fungus, and
- a chimeric DNA sequence capable of encoding a plant selectable marker allowing selection of transformed ancestor cells when said selectable marker is present therein, and

(b) regenerating said ancestor cells into plants under conditions favouring ancestor cells which have the said selectable marker, and

(c) identifying a plant which produces a protein according to claim 1, thereby reducing the susceptibility of said plant to infection by said fungus.

Preferred according to the invention is a method characterised in that step (a) is performed using an *Agrobacterium tumefaciens* strain capable of T-DNA transfer to plant cells and which harbours the said chimeric DNA cloned into binary vector pMOG800; another preferred method is when step (b) is performed in the presence of an antibiotic favouring cells which have a neomycin phosphotransferase.

The invention further provides an antifungal composition comprising a protein according to the invention and a suitable carrier.

An antibody, capable of reacting with an N-terminal fragment of a protein according to the invention, preferably to the peptide represented by SEQIDNO: 6, is also provided. The antibody is suitably used to detect expression levels of chimeric DNA according to the invention in host cells and multicellular hosts, preferably plants, capable of producing a protein according to the invention.

The invention also provides a nucleic acid sequence obtainable from a gene encoding a protein according to the invention, said nucleic acid sequence having tissue-specific transcriptional regulatory activity in a plant. The invention specifically provides a nucleic acid sequence obtainable from the region upstream of the translational initiation site of said gene, preferably at least 500 nucleotides immediately upstream of the translational initiation site of said gene.

DESCRIPTION OF THE FIGURES

Figure 1: SDS-PAGE (12.5%) of the different purification steps of MS59 sunflower protein. Mw= molecular weight markers; 1= crude sunflower protein extract after gel filtration (G25); 2= protein fraction bound to cation exchange chromatography (S-sepharose); 3= pool of active fractions after cation exchange chromatography (Mono S); 4= flow through from hydrophobic interaction chromatography (phenyl superose); 5= active fractions after gel filtration.

Figure 2: SDS-PAGE (12.5%) of different fractions (number 6 to 16) of the gelfiltration (SD75) column. Fraction 10 to 15 was tested in 3 dilutions for growth inhibition on *Phytophthora infestans* (PANEL A) and on *Pythium ultimum* (PANEL B)

Figure 3: SDS-PAGE (12.5%) of fractions eluted from nine gel slices (lane 1 to 9) of a native PAGE in which a MS59 containing SD75 fraction (SD75 fraction 13)was separated. Right panel: SDS-PAGE (12.5%) with SD75 fraction 13 (L) and two fractions of elution experiment fraction 2 (with MS59) and fraction 5 (with a ~30 kD protein). Bottom panel: growth inhibition of *Phytophthora infestans* tested with elution fraction 1 to 6, with 5 µl and 1 µl added per well.

DETAILED DESCRIPTION OF THE INVENTION

The antifungal effect of the protein(s) of the invention has been demonstrated in *in vitro* assays for the following fungi; *Phytophthora infestans, Phytophthora cactorum, Phytophtora nicotianae, Fusarium oxysporum, Pythium ultimum, Pythium sylvaticum* and for *Pythium paroecandrum* for purposes of illustration. It will be clear, that the use of the protein(s) of the invention, or DNA encoding therefore, for use in a process of combating fungi is not limited to the mentioned fungi. There is no reason to assume that the protein(s) according to the invention do not possess antifungal activity against a far broader range of fungi than those tested here, especially in the class of Oomycetes.

Although the invention is illustrated in detail for transgenic tomato, tobacco, carrot, potato

and *Brassica napus* plants, it should be understood that any plant species that is subject to some form of fungal attack, especially from the fungi mentioned above, may be provided with one or more plant expressible gene constructs, which when expressed overproduce the protein(s) of the invention in said plant in order to decrease the rate of infectivity and/or the effects of such attack.

The invention can even be practiced in plant species that are presently not amenable for transformation, as the amenability of such species is just a matter of time and because transformation as such is of no relevance for the principles underlying the invention. Hence, plants for the purpose of this description shall include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants, be they for feed, food or industrial processing purposes; included are plants used for any agricultural or horticultural purpose including forestry and flower culture, as well as home gardening or indoor gardening, or other decorative purposes.

The protein according to the present invention may be obtained by isolating it from any suitable plant source material containing it. A particularly suitable source comprises leaves of the sunflower (*Helianthus*). The presence of antifungal proteins according to the invention in plant source material can readily be determined for any plant species by making plant extracts from those species and testing those extracts for the presence of antifungal activity using an *in vitro* antifungal assay as described herein, further fractionating the obtained samples by any suitable protein fractionation technique in conjunction with the *in vitro* assay until an antifungal fraction is obtained which comprises an approximately 59 kDa protein, internally denoted as MS59, which in isolated form shows antifungal activity. Especially, fractions may be tested for antifungal activity on Oomycetes, for example, *Phythophthora* or *Pythium ultimum* and the like, or other fungi, such as the Basidiomycetes, or other taxons.

Alternatively, antifungal proteins according to the invention may be obtained by cloning DNA comprising an open reading frame capable of encoding said protein, or the precursor thereof, linking said open reading frame to a transcriptional, and optionally a translational initiation and transcriptional termination region, inserting said DNA into a suitable host cell and allowing said host cell to produce said protein. Subsequently, the protein may be recovered from said host cells, preferably after secretion of the protein into the culture medium by said host cells. Alternatively, said host cells may be used directly in a process of combating fungal pathogens according to the invention as a pesticidal acceptable composition.

Host cells suitable for use in a process of obtaining a protein according to the invention may be selected from prokaryotic microbial hosts, such as bacteria *e.g. Agrobacterium, Bacillus,* Cyanobacteria, *E.coli* , *Pseudomonas,* and the like, as well as eukaryotic hosts including yeasts, *e.g. Saccharomyces cerevisiae,* fungi, *e.g. Trichoderma* and plant cells, including protoplasts.

In a method of retarding the growth of the fungus *Phytophthora infestans* on plant leaves, characterised in that hyphae thereof, or spores thereof, are caused to be contacted with a protein produced from a host cell, host cells may suitably be selected from any species routinely used as biological fungicides.

Although the invention is set out in more detail using *Phytophthora infestans* as an example, it will be clear that proteins according to the invention may be tested for antifungal activity other than anti-*Phytophthora* activity using an antifungal assay similar to that described in the present specification. Suitable antifungal assays have been described for several other fungi in European patent application 440 304 A1.

The word protein means a sequence of amino acids connected trough peptide bonds. Polypeptides or peptides are also considered to be proteins. Muteins of the protein of the invention are proteins that are obtained from the proteins depicted in the sequence listing by replacing, adding and/or deleting one or more amino acids, while still retaining their antifungal activity. Such muteins can readily be made by protein engineering *in vivo*, e.g. by changing the open reading frame capable of encoding the antifungal protein such that the amino acid sequence is thereby affected. As long as the changes in the amino acid sequences do not altogether abolish the antifungal activity such muteins are embraced in the present invention.

The present invention provides a chimeric DNA sequence which comprises an open reading frame capable of encoding a protein according to the invention. The expression chimeric DNA sequence shall mean to comprise any DNA sequence which comprises DNA sequences not naturally found in nature. For instance, chimeric DNA shall mean to comprise DNA comprising the said open reading frame in a non-natural location of the plant genome, notwithstanding the fact that said plant genome normally contains a copy of the said open reading frame in its natural chromosomal location. Similarly, the said open reading frame may be incorporated in the plant genome wherein it is not naturally found, or in a replicon or vector where it is not naturally found, such as a bacterial plasmid or a viral vector. Chimeric DNA shall not be limited to DNA molecules which are replicable in a host, but shall also mean to comprise DNA capable of being ligated into a replicon, for instance by virtue of specific adaptor sequences, physically linked to the open reading frame according to the invention. The open reading frame may or may not be linked to its natural upstream and downstream regulatory elements.

The open reading frame may be derived from a genomic library. In this latter it may contain one or more introns separating the exons making up the open reading frame that encodes a protein according to the invention. The open reading frame may also be encoded by one uninterrupted exon, or by a cDNA to the mRNA encoding a protein according to the invention. Open reading frames according to the invention also comprise those in which one or more introns have been artificially removed or added. Each of these variants is embraced by the present invention.

In order to be capable of being expressed in a host cell a chimeric DNA according to the invention will usually be provided with regulatory elements enabling it to be recognised by the biochemical machinery of the host and allowing for the open reading frame to be transcribed and/or translated in the host. It will usually comprise a transcriptional initiation region which may be suitably derived from any gene capable of being expressed in the host cell of choice, as well as a translational initiation region for ribosome recognition and attachment. In eukaryotic cells, an expression cassette usually comprises in addition a transcriptional termination region located downstream of said open

Although some of the embodiments of the invention may not be practicable at present, *e.g.* because some plant species are as yet recalcitrant to genetic transformation, the practicing of the invention in such plant species is merely a matter of time and not a matter of principle, because the amenability to genetic transformation as such is of no relevance to the underlying embodiment of the invention.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell, as long as the cells are capable of being regenerated into whole plants. Methods may suitably be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, 1982, Nature 296, 72-74; Negrutiu I. *et al*, June 1987, Plant Mol. Biol. 8, 363-373), electroporation of protoplasts (Shillito R.D. *et al.*, 1985 Bio/Technol. 3, 1099-1102), microinjection into plant material (Crossway A. *et al.*, 1986, Mol. Gen. Genet. 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. *et al.*, 1987, Nature 327, 70), infection with (non-integrative) viruses and the like. A preferred method according to the invention comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838). Tomato transformation is preferably done essentially as described by Van Roekel et al. (Van Roekel, J.S.C., Damm, B., Melchers, L.S., Hoekema, A. (1993). Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Reports, 12, 644-647). Potato transformation is preferably done essentially as described by Hoekema et al. (Hoekema, A., Huisman, M.J., Molendijk, L., van den Elzen, P.J.M., and Cornelissen, B.J.C. (1989). The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio/Technology 7, 273-278).
Generally, after transformation plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant expressible genes co-transferred with the nucleic acid sequence encoding the protein according to the invention, whereafter the transformed material is regenerated into a whole plant.

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Presently, preferred methods for transformation of monocots are microprojectile bombardment of embryos, explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, *et al*, 1989, Nature 338, 274-276). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus bar*-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for

the establishment of the embryogenic suspension cultures (Vasil, 1990 Bio/Technol. 8, 429-434). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as corn are also amenable to DNA transfer by *Agrobacterium* strains (*vide* WO 94/00977; EP 0 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434).

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the chimeric DNA according to the invention, copy number and/or genomic organization. In addition, or alternatively, expression levels of the newly introduced DNA may be undertaken, using Northern and/or Western analysis, techniques well known to persons having ordinary skill in the art. After the initial analysis, which is optional, transformed plants showing the desired copy number and expression level of the newly introduced chimeric DNA according to the invention may be tested for resistance levels against a pathogen susceptible to the protein according to the invention, such as *Phytophthora infestans*. Alternatively, the selected plants may be subjected to another round of transformation, for instance to introduce further genes, such as genes encoding chitinases, glucanases, osmotins, magainins or the like, in order to enhance resistance levels, or broaden the resistance to other fungi found not to be susceptible to the protein according to the invention in an *in vitro* assay as described herein.

Other evaluations may include the testing of fungal resistance under field conditions, checking fertility, yield, and other characteristics. Such testing is now routinely performed by persons having ordinary skill in the art.

Following such evaluations, the transformed plants may be grown directly, but usually they may be used as parental lines in the breeding of new varieties or in the creation of hybrids and the like.

Many plant proteins exhibit antifungal effects, some however do not do so as such, but yield a significant synergistic antifungal effect if used in combination with other plant proteins. In European Patent Application 440 304 A1 it was disclosed that simultaneous relative over-expression of a plant expressible glucanase gene in conjunction with a basic chitinase from tobacco in transgenic plants results in a higher level of resistance to fungi than in plants expressing a plant expressible class-I chitinase alone.

Both chitinases, glucanases, osmotins, magainins and the new antifungal protein according to the invention accumulate in infected plant tissues upon an incompatible pathogen-plant interaction. From this observation and the fact that several proteins are found to synergise each others antifungal effects, we envision, that the antifungal protein according to the invention may be suitably used in conjunction with other proteins that are associated with pathogen resistance.

Examples of proteins that may be used in combination with the proteins according to the invention include, but are not limited to, β-1,3-glucanases and chitinases which are obtainable from barley (Swegle M. *et al.*, 1989, Plant Mol. Biol. 12, 403-412; Balance G.M. *et al.*, 1976, Can. J. Plant Sci. 56, 459-466 ; Hoj P.B. *et al.*, 1988, FEBS Lett. 230, 67-71; Hoj P.B. *et al.*, 1989, Plant

Mol. Biol. 13, 31-42 1989), bean (Boller T. *et al.*, 1983, Planta 157, 22-31; Broglie K.E. *et al.* 1986, Proc. Natl. Acad. Sci. USA 83, 6820-6824; Vögeli U. *et al.*, 1988 Planta 174, 364-372); Mauch F. & Staehelin L.A., 1989, Plant Cell 1, 447-457); cucumber (Motraux J.P. & Boller T. (1986), Physiol. Mol. Plant Pathol. 28, 161-169); leek (Spanu P. *et al.*, 1989, Planta 177, 447-455); maize (Nasser W. *et al.*, 1988, Plant Mol. Biol. 11, 529-538), oat (Fink W. *et al.*, 1988, Plant Physiol. 88, 270-275), pea (Mauch F. *et al.* 1984, Plant Physiol. 76, 607-611; Mauch F. *et al.*, 1988, Plant Physiol. 87, 325-333), poplar (Parsons, T.J. *et al*, 1989, Proc. Natl. Acad. Sci. USA 86, 7895-7899), potato (Gaynor J.J. 1988, Nucl. Acids Res. 16, 5210; Kombrink E. *et al.* 1988, Proc. Natl. Acad. Sci. USA 85, 782-786; Laflamme D. and Roxby R., 1989, Plant Mol. Biol. 13, 249-250), tobacco (*e.g.* Legrand M. *et al.* 1987, Proc. Natl. Acad. Sci. USA 84, 6750-6754; Shinshi H. *et al.* 1987, Proc. Natl. Acad. Sci. USA 84, 89-93), tomato (Joosten M.H.A. & De Wit P.J.G.M. 1989, Plant Physiol. 89, 945-951), wheat (Molano J. *et al.*, 1979, J. Biol. Chem. 254, 4901-4907), and the like.

To obtain transgenic plants capable of constitutively expressing more than one chimeric gene, a number of alternatives are available including the following:

A. The use of DNA, *e.g* a T-DNA on a binary plasmid, with a number of modified genes physically coupled to a selectable marker gene. The advantage of this method is that the chimeric genes are physically coupled and therefore migrate as a single Mendelian locus.

B. Cross-pollination of transgenic plants each already capable of expressing one or more chimeric genes, preferably coupled to a selectable marker gene, with pollen from a transgenic plant which contains one or more chimeric genes coupled to another selectable marker. Afterwards the seed, which is obtained by this crossing, maybe selected on the basis of the presence of the two selectable markers, or on the basis of the presence of the chimeric genes themselves. The plants obtained from the selected seeds can afterwards be used for further crossing. In principle the chimeric genes are not on a single locus and the genes may therefore segregate as independent loci.

C. The use of a number of a plurality chimeric DNA molecules, *e.g.* plasmids, each having one or more chimeric genes and a selectable marker. If the frequency of co-transformation is high, then selection on the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.

D. Consecutive transformation of transgenic plants already containing a first, second, (etc), chimeric gene with new chimeric DNA, optionally comprising a selectable marker gene. As in method B,the chimeric genes are in principle not on a single locus and the chimeric genes may therefore segregate as independent loci.

E. Combinations of the above mentioned strategies.

The actual strategy may depend on several considerations as maybe easily determined such as the purpose of the parental lines (direct growing, use in a breeding programme, use to produce hybrids) but is not critical with respect to the described invention.

In this context it should be emphasised that plants already containing chimeric DNA capable of encoding antifungal proteins may form a suitable genetic background for introducing chimeric DNA according to the invention, for instance in order to enhance resistance levels, or broaden the

resistance. The cloning of other genes corresponding to proteins that can suitably be used in combination with DNA, and the obtention of transgenic plants, capable of relatively over-expressing same, as well as the assessment of their effect on pathogen resistance *in planta* , is now within the scope of the ordinary skilled person in the art.

The obtention of transgenic plants capable of expressing, or relatively over-expressing, proteins according to the invention is a preferred method for counteracting the damages caused by fungi, such as Oomycetes like *Phytophthora infestans*, as will be clear from the above description. However, the invention is not limited thereto. The invention clearly envisions also the use of the proteins according to the invention as such, preferably in the form of a fungicidal composition. Fungicidal composition include those in which the protein is formulated as such, but also in the form of host cells, such as bacterial cells, capable of producing the protein thereby causing the pathogen to be contacted with the protein. Suitable host cells may for instance be selected from harmless bacteria and fungi, preferably those that are capable of colonising roots and/or leaves of plants. Example of bacterial hosts that may be used in a method according to the invention are strains of *Agrobacterium, Arthrobacter, Azospyrillum, Pseudomonas, Rhizobacterium,* and the like, optionally after having been made suitable for that purpose.

Compositions containing antifungal proteins according to the invention may comprise in addition thereto, osmotin-like proteins as defined in (WO91/18984). Independently, the invention provides antifungal compositions which further comprise inhibitory agents such as classical fungal antibiotics, SAFPs and chemical fungicides such as polyoxines, nikkomycines, carboxymides, aromatic carbohydrates, carboxines, morpholines, inhibitors of sterol biosynthesis, organophosphorus compounds, enzymes such as glucanases, chitinases, lysozymes and the like. Either per se, or in combination with other active constituents, the antifungal protein of the invention should be applied in concentrations between 1 ng/ml and 1 mg/ml, preferably between 2 ng/ml and 0.1 mg/ml, within pH boundaries of 3.0 and 9.0. In general it is desired to use buffered preparations, *e.g.* phosphate buffers between 1mM and 1M, preferably between 10 mM and 100mM, in particular between 15 and 50 mM, whereby in case of low buffer concentrations it is desired to add a salt to increase ionic strength, preferably NaCl in concentrations between 1 mM and 1M, preferably 10 mM and 100 mM.

Plants, or parts thereof, which relatively over-express a protein according to the invention, including plant varieties, with improved resistance against fungal diseases, especially disease caused by Oomycetes like *Phytophthora* and *Pythium* may be grown in the field, in the greenhouse, or at home or elsewhere. Plants or edible parts thereof may be used for animal feed or human consumption, or may be processed for food, feed or other purposes in any form of agriculture or industry. Agriculture shall mean to include horticulture, arboriculture, flower culture, and the like. Industries which may benefit from plant material according to the invention include but are not limited to the pharmaceutical industry, the paper and pulp manufacturing industry, sugar manufacturing industry, feed and food industry, enzyme manufacturers and the like.
The advantages of the plants, or parts thereof, according to the invention are the decreased need

for fungicide treatment, thus lowering costs of material, labour, and environmental pollution, or prolonging shelf-life of products (e.g. fruit, seed, and the like) of such plants. Plants for the purpose of this invention shall mean multicellular organisms capable of photosynthesis, and subject to some form of fungal disease. They shall at least include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants.

The phrase "plants which relatively over-express a protein" shall mean plants which contain cells expressing a transgene-encoded protein which is either not naturally present in said plant, or if it is present by virtue of an endogenous gene encoding an identical protein, not in the same quantity, or not in the same cells, compartments of cells, tissues or organs of the plant. It is known for instance that normally intracellular proteins may be targeted to the apoplastic space.

According to another aspect of the invention the regulatory region of a plant gene coding for the antifungal protein of the invention may be used to express other heterologous sequences under the control thereof. The use of a regulatory element of at least 1000 bp directly upstream of the gene coding region is sufficient for obtaining expression of any heterologous sequence.

Heterologous sequences in this respect means gene regions not naturally associated to said regulatory region, and they comprise both different gene coding regions, as well as antisense antisense gene regions.

Heterologous coding sequences that may be advantageously expressed in the vascular tissue comprise those coding for antipathogenic proteins, *e.g.* insecticidal, bactericidal, fungicidal, and nematicidal proteins. In such a strategy it may prove exceptionally advantageous to select a protein with activity against a pathogen or pest which has a preference for phloem as source of nutrients (*e.g.* aphids), or as entrance to invade the plant. Examples are extensin, lectin, or lipoxidase against aphids (See WO93/04177). Assuming that the regulatory region according to the invention is active in xylem, chitinases and glucanases may be expressed under the control of said regulatory region to combat *Fusarium, Verticillium, Alternaria* and *Ceratocystus* species.

The use of the regulatory region according to the invention may also be used advantageously to regulate or control phloem transport processes. Numerous other applications will readily occur to those of skill in the art.

The expression of part of (part of) an endogenous gene in the antisense orientation (such as disclosed in EP 0 233 399 A), can effectively down-regulate expression of said endogenous gene, with interesting applications. Moreover, the gene encoding the antifungal protein according to the invention itself may be down-regulated using the antisense approach which may help establishing the nature and function of the protein. The regions responsible for tissue-specific expression may be unravelled further using the GUS-marker in a way analogous to the way illustrated herein.

The following state of the art may be taken into consideration, especially as illustrating the general level of skill in the art to which this invention pertains.

EP-A 392 225 A2; EP-A 440 304 A1; EP-A 460 753 A2; WO90/07001 A1; US Patent 4,940,840.

Evaluation of transgenic plants

Subsequently transformed plants are evaluated for the presence of the desired properties and/or the extent to which the desired properties are expressed. A first evaluation may include the level of expression of the newly introduced genes, the level of fungal resistance of the transformed plants, stable heritability of the desired properties, field trials and the like.

Secondly, if desirable, the transformed plants can be crossbred with other varieties, for instance varieties of higher commercial value or varieties in which other desired characteristics have already been introduced, or used for the creation of hybrid seeds, or be subject to another round of transformation and the like.

Synergy

The combination of the sunflower (MS59) antifungal protein according to the instant invention and other antifungal proteins of plant or microbial source are predicted to show a drastic synergistic antifungal effect. Similar synergistic antifungal effects were shown if combinations of antifungal CBPs or Chi-V are combined with either ß-1,3-glucanases or chitinases from other plant origins. Apparently, the synergizing effect of combinations of pathogen induced proteins is a more general phenomenon that has important consequences for the engineering of fungal resistant plants.

Plants, or parts thereof of commercial interest, with improved resistance against phytopathogenic fungi can be grown in the field or in greenhouses, and subsequently be used for animal feed, direct consumption by humans, for prolonged storage, used in food- or other industrial processing, and the like. The advantages of the plants, or parts thereof, according to the invention are the decreased need for fungicide treatment, thus lowering costs of material, labour, and environmental pollution, or prolonged shelf-life of products (e.g. fruit, seed, and the like) of such plants.

EXPERIMENTAL

Standard methods for the isolation, manipulation and amplification of DNA, as well as suitable vectors for replication of recombinant DNA, suitable bacterium strains, selection markers, media and the like are described for instance in Maniatis *et al.*, molecular cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press; DNA Cloning: Volumes I and II (D.N. Glover ed. 1985); and in: From Genes To Clones (E.-L. Winnacker ed. 1987).

In vitro antifungal assay on Oomycetes.

The antifungal activity was monitored during purification in a microtiter plate assay using the fungi *Phytophthora infestans* and *Pythium ultimum* according to Woloshuk *et al.*, 1991. In each well of a 24-well microtiter dish 250 µl potato dextrose agar (PDA) was pipetted. Fungal spores in the case of *Phytophthora infestans* and hyphal fragments in the case of *Pythium ultimum* were suspended in water and 400-600 spores or 200 fragments in 50 µl were added to the wells. Subsequently 100 µl filter sterilized (0.22 µm filter) protein solution (in 50 mM MES, pH 6.0) was added. Microtiter dishes were wrapped with Parafilm and incubated at room temperature. At several timepoints after the initiation of incubation the fungus was monitored microscopically for effects of the added protein. After 2-3 days the mycelium of the growing fungus in the wells was stained with lactophenol cotton blue and the extent of growth was estimated.
GI: growth inhibition; a scale of 0 - 4 is used, 0 = no visible inhibition, 1 = weak inhibition (0 to 30%) inhibition, 2 = moderate (30 to 60%) inhibition, 3 = strong (60 to 90%) inhibition, 4 = very strong (100%) inhibition.

EXAMPLE 1

Purification of an antifungal protein MS59 from sunflower induced with salicylic acid.
Leaves of 7 to 8 weeks old sunflower (*Helianthus annuus* cv. zebulon) plants were sprayed daily for 5 times with 10 mM sodium salicylate. After 3 hours the plants were extensively rinsed with water to remove the sodium salicylate. Three days after the final spray leaves (400 gram) were harvested into liquid nitrogen and homogenized at 4°C in 500 ml 0.5 M NaOAc pH5.2, and 4 gram active carbon, using a Waring blendor. The homogenate was filtered over four layers of cheese cloth and subsequently the filtrate was centrifuged for 50 minutes at 20,000 g at 4°C and desalted by passage through a Sephadex G25 column (medium course; Pharmacia), length 60 cm, diameter 11.5 cm, equilibrated in 40 mM NaOAc pH5.2. The desalted protein solution was stored overnight at 4°C and subsequently centrifuged for 45 minutes at 20,000 g at 4°C. The supernatant was passed through a S-sephadex (Fast-flow, Pharmacia) column, length 5 cm, diameter 5 cm, which was equilibrated with 40 mM NaOAc pH 5.2. The column was washed with the above mentioned buffer (flow rate 400 to 500 ml/hr) until the OD_{280} dropped to zero. The bound proteins were eluted

using a 400 mM NaCl in 200 ml of the above mentioned buffer.

After dialysis against 50 mM MES pH 6.0 the eluate was analyzed for antifungal activity. Antifungal activity was monitored in a microtiter plate assay using the fungus *Phytophthora infestans* and *Pythium ultimum*. See above for details concerning *in vitro* assaying. Subsequently, cationexchange chromatography was reapplied whereby the eluate was passed through an FPLC Mono-S HR 5/5 (Pharmacia) and eluted with a linear gradient from 0 to 400 mM NaCl. All fractions were analyzed by electrophoresis (Laemmli (1970), Nature 227:680-685) using a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS), using prestained molecular weight markers (15-105 kDa) as reference. Additionally, of all fractions antifungal activity towards *Phytophthora infestans* and *Pythium ultimum* was monitored. Antifungal activity eluted from the column between 45-60 mM NaCl and in all active fractions a 59 kD band was visible. Fractions containing the antifungal activity were pooled and dialysed to 1 M ammonium sulphate in 50 mM potassium phosphate, pH 7. The pool was subjected to hydrophobic interaction chromatography, whereby the sample was applied to an FPLC Phenyl Superose HR 5/5 (Pharmacia) equilibrated in the same buffer and eluted with a linear decreasing gradient from 1 to 0 M ammonium sulphate in 50 mM potassium phosphate, pH 7. As above again all fractions were analyzed on SDS-PAGE and monitored for antifungal activity. Also the pool of proteins was thus analyzed not capable of binding to this column (Flow Through, FT) at the conditions chosen here. Antifungal was present most abundantly in the FT and secondly also in the fractions eluting between 0.76 and 0.45 mM ammonium sulphate. In both cases a 59 kD protein was visible on SDS-PAGE. FT and the gradient fractions were separately dialysed to 50 mM MES, 0.2 M NaCl and separately chromatographed on a FPLC Superdex 75 HR 10/30 column (Pharmacia) equilibrated to the same buffer. Proteins elute from this column according to their molecular size. In both cases again the presence of a 59 kD protein coincided with antifungal activity towards *Phytophthora infestans* and *Pythium ultimum* as judged from SDS-PAGE and *in vitro* antifungal assays. The 59 kD protein present in the FT of the hydrophobic interaction column was most abundant and termed MS59 and its purification is visualized in Figure 1. Results of its separation over the gelfiltration column and subsequent analysis both on SDS-PAGE and on *Phytophthora infestans* is shown in Figure 2. Several characteristics (antifungal activity, chromatographical properties, molecular mass) of the gradient protein and MS59 indicate that the two proteins are very similar.

To characterize MS59 further its amino acid sequence was partially determined. Therefore, MS59 was separated in the presence of 0.1 mM thioglycolate in the upper reservoir buffer and SDS on a 12.5% polyacrylamide gel, which was prerun for 2 hours at 50 V with 0.05 mM glutathion in the upper reservoir buffer. The gel was stained with 5% (w/v) Serva Blue G in 45% (v/v) methanol and 10% acetic acid for 30 minutes and destained in 20% (v/v) acetic acid for 30 minutes and the 59 kDa band was cut out and sequenced using Edman degradation on an Applied Biosystems 477A protein sequencer according to the protocol provided by the manufacturer. N-terminal amino acid sequencing of MS59 revealed that the N-terminus was blocked. To obtain internal sequences, MS59 was digested with trypsin. Trypsin cleaves protein at arginine and lysine residues. The digestion

products were separated on a reversed-phase column and analyzed by Edman degradation. Two tryptic fragments were sequenced: Pep1 and Pep2. Of Pep1 25 amino acid residues were identified: S-I-N-V-D-I-E-Q-E-T-A-W-V-Q-A-G-A-T-L-G-E-V-Y-Y-R (SEQIDNO: 1).
The amino acid sequence is given using the one-letter code. Of Pep2 a further 25 amino acid residues were identified: D-P-S-F-P-I-T-G-E-V-Y-T-P-G-(?)-S-S-F-P-T-V-L-Q-N-Y (SEQIDNO: 2).
The amino acid residue between brackets could not be identified unambiguously.

Polyclonal antibodies were raised against MS59. Firstly, the whole MS59 protein was used to raise antibodies. Therefore, MS59 was separated on SDS-polyacrylamide gels and the MS59 band was cut out after visualization. The band (approximately 100 µg protein) was dehydrated in EtOH, dialysed extensively against phosphate buffered saline (PBS), ground in a mortar and injected into a rabbit. After one month the rabbit was boostered every 2 weeks with 50 µg MS59 protein. After 15 weeks the rabbit was sacrificed. The antiserum was further purified on a horse radish peroxidase (HRP) column, since the antiserum was cross-reactive with a lot of probably glycosylated proteins. After purification the antiserum specifically recognized a protein band of 59 kDa and showed some minor cross reaction with a low number of other proteins.
In a second approach peptides of MS59, which were synthesized according to the amino acid sequence of SEQIDNO:1 and SEQIDNO:2, or parts thereof, coupled to bovine serum albumine were used to raise antiserum. This antiserum, which did not need a purification on a HRP column, specifically recognized a protein band of 59 kDa.

EXAMPLE 2

Elution of antifungal protein from native PAGE and subsequent testing

It is obvious from Figure 1 that MS59 is not completely pure. To further ensure that indeed the 59 kDa protein is responsible for the observed antifungal activity, the fraction containing the peak amount of 59 kDa was electrophoresed on a native gel, using the same system as described above however without SDS and without boiling the samples before loading. The gel lane was sliced in 0.5 cm horizontal pieces and each piece was eluted individually for 48 hours in 50 mM Mes, pH 6. After centrifugation the resulting supernatant was analyzed both on SDS-PAGE and *in vitro* for antifungal activity. Results are shown in Figure 3. Only in those fractions containing MS59, was antifungal activity observed against *Phytophthora infestans* and *Pythium ultimum*.

EXAMPLE 3

In vitro antifungal assays on non-Oocymetes.

All fungi were cultured on potato dextrose agar (Difco) at 25°C, except *Botrytis cinerea* and *Phoma lingam* which were grown on oat meal agar (Difco) at 25°C. *Phytophthora infestans* was grown on rye agar at 18°C in the dark (Caten and Jinks, 1968). *Botrytis cinerea* and *Phoma*

lingam were cultivated under UV. Spores of sporulating fungi were harvested by flooding the agar plates with water. The spore concentration was adjusted to 10,000 sp/mL. In the case of *Rhizoctonia solani* and *Pythium ultimum* liquid shake cultures were grown in potato dextrose broth at 25°C. To prepare inoculum from these shake cultures, mycelium was harvested and vortexed for 1 minute. After passage through a fine sieve, inoculum density was adjusted to 2500 - 5000 fragments, of 1 to 3 cells each, per mL.

In case of sporulating fungi, all were tested both with and without pregerminating the spores before application of the protein samples. In case of non-sporulating fungi, hyphal fragments were used. Fractions eluting from the Mono-S, pH 6 were assayed for the presence of antifungal activity. As positive control *Phytophthora infestans* was tested. The peak of MS59 is located in fraction 4. Results are shown in Table 1.

TABLE 1

Antifungal effects of MS59 containing fractions from Mono-S, pH 6

fungus	spore stage *)	fraction number 1	2	3	4	5	6	7	8
Botrytis cinerea	spore	0	0	0	0	0	0	0	0
	germl.	0	0	0	0	0	0	0	0
Fusarium oxysporum	spore	0	0	0	0	0	0	0	0
	germl.	2	2	2	2	3	3.5	3.5	3.5
Fusarium solani	spore	0	0	0	0	0	0	0	0
Alternaria solani	spore	0	0	0	0	0	0	0	0
	germl.	0	0	0	0	0	0	0	0
Phoma lingam	spore	0	0	0	0	0	0	0	0
	germl.	0	0	0	0	0	0	0	0
Colletotrichum coccodes	spore	0	0	0	0	0	0	0	0
	germl.	0	0	0	0	0	0	0	0
Rhizoctonia solani	hyph.	0	0	0	0	0	0	0	0
Phytophthora infestans	spore	0	2	2	4	3.5	2	1	0
Phytophthora nicotianae	hyph	0	1	2	4	4	2	1	0
Phytophthora cactorum	hyph	0	0	2	4	4	1	1	0
Pythium ultimum	hyph	0	0	0	4	4	0	0	0
Pythium sylvaticum	hyph	0	0	0	2	1	0	0	0
Pythium paroecandrum	hyph	0	0	0	2	2	0	0	0

*) spore = no pregermination, germl = germination until the germtube is 3-5 times the length of the spore, hyph. = hyphal fragments were used as starting inoculum.

GI: growth inhibition; a scale of 0 - 4 is used, 0 = no visible growth inhibition, 1 = weak (0 to 30%) inhibition, 2 = moderate (30 to 60%) inhibition, 3 = strong (60 to 90%) inhibition, 4 = very strong (100%) inhibition.

As can be seen *Phytophthora* and *Pythium* spp., appeared very sensitive to MS59, indicating the specificity of MS59 for Oomycetes.

EXAMPLE 4

Identification and characterization of genes homologous to the deduced MS59 nucleotide sequence.

Based on the amino acid sequences of pep1 (a.a. 12 to 22 of SEQIDNO: 1) and pep2 (a.a. 2 to 12 of SEQIDNO: 2), primers were designated for PCR. Genomic DNA was isolated from sunflower cv. Zebulon and PCR primers 4 (5'AAC TTC TCC AIAG IGT IGC ICC IGC TTG IAC CCA3', SEQIDNO: 3) and 5 (5'GAT CCI TCT TTC CCI ATT ACT GGI GAG GTT TA3', SEQIDNO: 4) were used to amplify a 354 bp DNA fragment from the sunflower genome with PCR. PCR products corresponding to this fragment size were cloned. Sequence analysis of the product revealed the presence of an uninterrupted Open Reading Frame (ORF) of which the first and last stretch of amino acids corresponded with the amino acid sequences of SEQIDNO: 1 and SEQIDNO: 2. The ORF (sense strand indicated in SEQIDNO: 5)-encoded amino acid sequence is SEQIDNO: 6. Several clones sequenced contained point mutations, varying from 1 to 4 in this PCR fragment. All but one of these mutations were silent mutations (nucleotide nr 57 T to C, nucleotide nr 63 C to A, nucleotide nr 225 A to G) which therefore did not alter amino acid sequences encoded. One clone however did contain a point mutation (nucleotide nr 203 G to A) which altered the amino acid sequence at amino acid 68 from Arg to Lys.

A southern blot of sunflower genomic DNA, probed with SEQIDNO: 5 indicated the existence of multiple homologous sequences in the genome. Using SphI, 6 bands were detected, EcoRV 5 bands, SpeI 3 bands and NdeI 4 bands. With other enzymes 3-4 bands were previously discerned. This analysis suggests the existence of 3 genes with (partial) homology to the ms59 sequences.

New PCR primers were developed based on the non-variable areas between the original PCR primer sequences. Primers: for 3' RACE: 5' CAG GCA GCT GTG GTT TGT GGC 3' (SEQIDNO:7), for 5' RACE: 5' GTC CAC AAT GAA GAA GGG TTG 3' (SEQIDNO: 8) and for nested 3'RACE: 5' ACG

TAG ATA TCG AAC AAG AAA CCG C 3' (SEQIDNO: 9).

Poly(A)-containing RNA was isolated from sunflower leaf material that was induced by spraying 5 times with a 10 mM sodium salicylate solution. cDNA was prepared and 5' and 3' RACE PCR reactions were performed as described in the instructions of the Marathon™ kit (Clontech Laboratories, Inc., Palo Alto,CA). DNA sequences and amino acid sequences of ORF encoded thereby of different clones isolated are shown in SEQIDNO: 10, SEQIDNO: 11, SEQIDNO: 12, SEQIDNO: 13, SEQIDNO: 14, SEQIDNO: 15.

cDNA clones are isolated which encode all of the Open Reading Frame including a putative signal peptide followed by an approximately 59 kD protein, and 5' and 3' UTRs (untranslated regions). Using inverse-PCR reactions on sunflower genomic DNA we are able to isolate a fragment that coincides with the region surrounding the putative transcriptional start. Primer extension experiments will confirm the presence of a transcriptional start here.

EXAMPLE 5

Tailoring a MS59 clone for expression in transgenic plants

Fragments of various cDNA clones corresponding to the MS59 sequences are assembled into one large clone comprising the entire MS59 cDNA. Using a PCR reaction with Pfu DNA polymerase, we amplify the entire ORF, using the PCR primers to introduce a NcoI-compatible restriction site on the startcodon ATG and a suitable restriction enzyme recognition site just downstream of the stopcodon. The integrity of the DNA sequence is confirmed by sequencing. The entire ORF is linked to a constitutive promoter (35S CaMV) which allows high level protein expression in most parts of the plant. After the ORF a 3' untranslated region of the potato proteinase inhibitor II (Thornburg *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84, 744-748), which contains sequences needed for polyadenylation (An *et al.*, 1989, Plant Cell 1, 115-122), is introduced. The chimeric gene produced is introduced into binary vector pMOG800 (deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, under CBS 414.93, on August 12, 1993). The resulting clone pMOG1144 is introduced in *Agrobacterium tumefaciens* strain EHA105, suitable for transformation of target crops tomato and potato, strain MOG101 for transformation of tobacco and *Arabidopsis* and MOG301 for transformation of *Brassica napus*.

In addition to this single gene construct, two 4-gene construct are made designated;

a) pMOG1145, which contains the tobacco genes encoding class I ß-1,3-glucanase, class I chitinase and AP24 under control of heterologous promoters and the MS59 gene construct as described before, and

b) pMOG1146, which contains the tobacco genes encoding class I ß-1,3-glucanase, class I chitinase and 16 kDa basic PR-1 under control of heterologous promoters and the described MS59 gene construct. These four gene constructs are constructed in the binary vector pMOG800 and introduced into *Agrobacterium tumefaciens* strain EHA105 for plant transformation.

WI), and the correct insertion of the fragment is confirmed using DNA sequencing. Then, the plasmid is introduced into *E. coli* BL21 (DE3) pLysS (Novagen, Madison, WI). Small scale cultures (2 ml) of several colonies are then started of which half is induced by the addition of IPTG to 1mM final concentration. Total extracts from E.coli are run on SDS gels and analyzed by Coomassie Brilliant Blue staining.

Several clones exhibit strong overexpression of the MS59 related protein. A clone which has strong overexpression is selected for a large scale culture. Five hundred ml of LB supplemented with 0.4 mM glucose is inoculated with a culture of this *E. coli* and grown to an optical density of 0.5-0.7. Then, IPTG is added to a final concentration of 1 mM and protein production is allowed for 3 hours at 37°C. A large proportion of the MS59 protein will be found in the insoluble protein fraction, a small amount will appear soluble. The soluble protein is purified by means of the histidine tag covalently attached to the MS59 protein. An immobilized metal ion affinity column coupled to (Ni^+) is loaded with the extract containing soluble E. coli proteins. After washing, the MS59 protein is eluted with an imidazole-containing buffer. The MS59 protein is the predominant protein in this eluate. Digestion of the chimeric protein with the protease enterokinase leads to cleavage into two parts. The MS59 part is further purified and checked in fungal inhibition on *Phytophthora infestans* germlings. The insoluble protein fraction is washed repeatedly with high salt buffers containing mild detergents. The resulting insoluble protein preparation will contain mainly MS59 protein. This preparation is used for raising antibodies. Two rabbits (NZW) are injected at regular intervals with MS59 protein. Antiserum from these rabbits is tested on Western blots containing purified E.coli- and sunflower derived MS59. Both proteins will be readily detected by the antiserum using standard Western blot procedures.

EXAMPLE 8

Purification of MS59 transproteins from tobacco transgenics.

Transgenic tobacco plants are produced expressing MS59 constitutively. Levels of expression are determined using Western analysis. Extracts of the transgenic material are assayed for *in vitro* growth inhibitory activity against *Phytophthora infestans and Pythium ultimum*. The extracts are made by grinding up leaf tissue from transgenic plants in 50 mM NaOAc, pH=5.2. After repeated centrifugation, overnight incubation on ice and an additional centrifugation step, the supernatant is dialysed to 15 mM potassium phosphate + 20 mM sodium chloride, pH =6. After filter sterilisation, 100 µg protein in 100 µl dialysis buffer is added per well containing 250 µl PDA and 50 µl water containing 400-600 spores. Growth inhibition is scored after 3 to 4 days.

EXAMPLE 9

Introduction of the four genes construct containing Chi-I, Glu-I, AP24 and MS59 under control of a constitutive plant promoter, into tomato, potato, carrot, Brassica napus and Arabidopsis

Using *Agrobacterium* mediated transformation system binary construct pMOG1145 containing the genes encoding Chi-I, Glu-I, AP24 and MS59 or pMOG1146 containing the genes encoding Chi-I, Glu-I, bPR-1 and MS59 is introduced into different crop species including, tomato, potato, carrot, *Brassica napus* and Arabidopsis.
S1 progeny plants are tested in fungal infection assays.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MOGEN International nv
(B) STREET: Einsteinweg 97
(C) CITY: Leiden
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 2333 CB
(G) TELEPHONE: 31-(0)71-5258282
(H) TELEFAX: 31-(0)71-5221471

(ii) TITLE OF INVENTION: Antifungal proteins, DNA coding therefor, and hosts incorporating same.

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helianthus annuus
(B) STRAIN: cv. zebulon

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Ile Asn Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly
1 5 10 15

Ala Thr Leu Gly Glu Val Tyr Tyr Arg
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helianthus annuus
(B) STRAIN: cv. zebulon

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp Pro Ser Phe Pro Ile Thr Gly Glu Val Tyr Thr Pro Gly Xaa Ser
1 5 10 15

Ser Phe Pro Thr Val Leu Gln Asn Tyr
20 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /function= "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AACTTCTCCI AGIGTIGCIC CIGCTTGIAC CCA 33

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: YES

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /function= "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GATCCITCTT TCCCIATTAC TGGIGAGGTT TA 32

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helianthus annuus

(B) STRAIN: cv. zebulon

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..354

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
GAT CCG TCT TTC CCG ATT ACT GGG GAG GTT TAC ACT CCC GGA AAC TCA      48
Asp Pro Ser Phe Pro Ile Thr Gly Glu Val Tyr Thr Pro Gly Asn Ser
 1               5                  10                  15

TCT TTT CCT ACC GTC TTG CAA AAC TAC ATC CGA AAC CTT CGG TTC AAT      96
Ser Phe Pro Thr Val Leu Gln Asn Tyr Ile Arg Asn Leu Arg Phe Asn
            20                  25                  30

GAA ACT ACC ACA CCA AAA CCC TTT TTA ATC ATC ACA GCC GAA CAT GTT     144
Glu Thr Thr Thr Pro Lys Pro Phe Leu Ile Ile Thr Ala Glu His Val
        35                  40                  45

TCC CAC ATT CAG GCA GCT GTG GTT TGT GGC AAA CAA AAC CGG TTG CTA     192
Ser His Ile Gln Ala Ala Val Val Cys Gly Lys Gln Asn Arg Leu Leu
    50                  55                  60

CTG AAA ACC AGA AGC GGT GGT CAT GAT TAT GAA GGT CTT TCC TAC CTT     240
Leu Lys Thr Arg Ser Gly Gly His Asp Tyr Glu Gly Leu Ser Tyr Leu
65                  70                  75                  80

ACA AAC ACA AAC CAA CCC TTC TTC ATT GTG GAC ATG TTC AAT TTA AGG     288
Thr Asn Thr Asn Gln Pro Phe Phe Ile Val Asp Met Phe Asn Leu Arg
                85                  90                  95

TCC ATA AAC GTA GAT ATC GAA CAA GAA ACC GCA TGG GTC CAA GCC GGC     336
Ser Ile Asn Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly
            100                 105                 110

GCC ACC CTC GGA GAA GTT                                            354
Ala Thr Leu Gly Glu Val
        115
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Asp Pro Ser Phe Pro Ile Thr Gly Glu Val Tyr Thr Pro Gly Asn Ser
 1               5                  10                  15

Ser Phe Pro Thr Val Leu Gln Asn Tyr Ile Arg Asn Leu Arg Phe Asn
            20                  25                  30

Glu Thr Thr Thr Pro Lys Pro Phe Leu Ile Ile Thr Ala Glu His Val
        35                  40                  45

Ser His Ile Gln Ala Ala Val Val Cys Gly Lys Gln Asn Arg Leu Leu
    50                  55                  60

Leu Lys Thr Arg Ser Gly Gly His Asp Tyr Glu Gly Leu Ser Tyr Leu
65                  70                  75                  80

Thr Asn Thr Asn Gln Pro Phe Phe Ile Val Asp Met Phe Asn Leu Arg
                85                  90                  95

Ser Ile Asn Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly
            100                 105                 110

Ala Thr Leu Gly Glu Val
        115
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /function= "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGGCAGCTG TGGTTTGTGG C 21

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /function= "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTCCACAATG AAGAAGGGTT G 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /function= "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACGTAGATAT CGAACAAGAA ACCGC 25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..396

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
CAT CTT CTT TCA ACA TGT CAA ACT TCC ATT CTT ACT CTC CTT CTT CTC        48
His Leu Leu Ser Thr Cys Gln Thr Ser Ile Leu Thr Leu Leu Leu Leu
 1               5                  10                  15

TTG CTC TCA ACC CAA TCT TCT GCA ACT TCC CGT TCC ATT ACA GAT CGC        96
Leu Leu Ser Thr Gln Ser Ser Ala Thr Ser Arg Ser Ile Thr Asp Arg
            20                  25                  30

TTC ATT CAA TGT TTA CAC GAC CGG GCC GAC CCT TCA TTT CCG ATA ACC       144
Phe Ile Gln Cys Leu His Asp Arg Ala Asp Pro Ser Phe Pro Ile Thr
        35                  40                  45

GGA GAG GTT TAC ACT CCC GGA AAC TCA TCT TTT CCT ACC GTC TTG CAA       192
Gly Glu Val Tyr Thr Pro Gly Asn Ser Ser Phe Pro Thr Val Leu Gln
    50                  55                  60

AAC TAC ATC CGA AAC CTT CGG TTC AAT GAA ACT ACC ACA CCA AAA CCC       240
Asn Tyr Ile Arg Asn Leu Arg Phe Asn Glu Thr Thr Thr Pro Lys Pro
65                  70                  75                  80

TTT TTA ATC ATC ACA GCC GAA CAT GTT TCC CAC ATT CAG GCA GCT GTG       288
Phe Leu Ile Ile Thr Ala Glu His Val Ser His Ile Gln Ala Ala Val
                85                  90                  95

GTT TGT GGC AAA CAA AAC CGG TTG CTA CTG AAA ACC AGA AGC GGT GGT       336
Val Cys Gly Lys Gln Asn Arg Leu Leu Leu Lys Thr Arg Ser Gly Gly
            100                 105                 110

CAT GAT TAT GAA GGT CTT TCC TAC CTT ACA AAC ACA AAC CAA CCC TTC       384
His Asp Tyr Glu Gly Leu Ser Tyr Leu Thr Asn Thr Asn Gln Pro Phe
        115                 120                 125

TTC ATT GTG GAC                                                      396
Phe Ile Val Asp
    130
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 132 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
His Leu Leu Ser Thr Cys Gln Thr Ser Ile Leu Thr Leu Leu Leu Leu
 1               5                  10                  15

Leu Leu Ser Thr Gln Ser Ser Ala Thr Ser Arg Ser Ile Thr Asp Arg
            20                  25                  30

Phe Ile Gln Cys Leu His Asp Arg Ala Asp Pro Ser Phe Pro Ile Thr
        35                  40                  45

Gly Glu Val Tyr Thr Pro Gly Asn Ser Ser Phe Pro Thr Val Leu Gln
    50                  55                  60

Asn Tyr Ile Arg Asn Leu Arg Phe Asn Glu Thr Thr Thr Pro Lys Pro
65                  70                  75                  80

Phe Leu Ile Ile Thr Ala Glu His Val Ser His Ile Gln Ala Ala Val
                85                  90                  95

Val Cys Gly Lys Gln Asn Arg Leu Leu Leu Lys Thr Arg Ser Gly Gly
            100                 105                 110

His Asp Tyr Glu Gly Leu Ser Tyr Leu Thr Asn Thr Asn Gln Pro Phe
        115                 120                 125

Phe Ile Val Asp
    130
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 704 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 3..704

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
AC GTA GAT ATC GAA CAA GAA ACC GCA TGG GTC CAA GCC GGT GCG ACT          47
   Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly Ala Thr
     1               5                  10                  15

CTT GGT GAA GTG TAC TAT CGA ATA GCG GAG AAA AGT AAC AAG CAT GGT          95
Leu Gly Glu Val Tyr Tyr Arg Ile Ala Glu Lys Ser Asn Lys His Gly
                 20                  25                  30

TTT CCG GCA GGG GTT TGT CCA ACG GTT GGC GTT GGT GGG CAT TTT AGT         143
Phe Pro Ala Gly Val Cys Pro Thr Val Gly Val Gly Gly His Phe Ser
             35                  40                  45

GGT GGT GGG TAT GGT AAT TTG ATG AGA AAA TAT GGT TTG TCG GTT GAT         191
Gly Gly Gly Tyr Gly Asn Leu Met Arg Lys Tyr Gly Leu Ser Val Asp
         50                  55                  60

AAT ATT GTT GAT GCT CAA ATA ATA GAT GTG AAT GGC AAG CTT TTG GAT         239
Asn Ile Val Asp Ala Gln Ile Ile Asp Val Asn Gly Lys Leu Leu Asp
     65                  70                  75

CGA AAG AGT ATG GGT GAG GAT TTG TTT TGG GCG ATC ACC GGC GGT GGT         287
Arg Lys Ser Met Gly Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly
 80                  85                  90                  95

GGT GTT AGT TTT GGT GTG GTT CTA GCC TAC AAA ATC AAA CTA GTT CGT         335
Gly Val Ser Phe Gly Val Val Leu Ala Tyr Lys Ile Lys Leu Val Arg
                100                 105                 110

GTT CCG GAG GTT GTG ACC GTG TTT ACC ATT GAA AGA AGA GAG GAA CAA         383
Val Pro Glu Val Val Thr Val Phe Thr Ile Glu Arg Arg Glu Glu Gln
            115                 120                 125

AAC CTC AGC ACC ATC GCG GAA CGA TGG GTA CAA GTT GCT GAT AAG CTA         431
Asn Leu Ser Thr Ile Ala Glu Arg Trp Val Gln Val Ala Asp Lys Leu
        130                 135                 140

GAT AGA GAT CTT TTC CTT CGA ATG ACC TTT AGT GTC ATA AAC GAT ACC         479
Asp Arg Asp Leu Phe Leu Arg Met Thr Phe Ser Val Ile Asn Asp Thr
    145                 150                 155

AAC GGT GGA AAG ACA GTC CGT GCT ATC TTT CCA ACG TTG TAC CTT GGA         527
Asn Gly Gly Lys Thr Val Arg Ala Ile Phe Pro Thr Leu Tyr Leu Gly
160                 165                 170                 175

AAC TCG AGG AAT CTT GTT ACA CTT TTG AAT AAA GAT TTC CCC GAG TTA         575
Asn Ser Arg Asn Leu Val Thr Leu Leu Asn Lys Asp Phe Pro Glu Leu
                180                 185                 190
```

```
GGG TTG CAA GAA TCG GAT TGT ACT GAA ATG AGT TGG GTT GAG TCT GTG        623
Gly Leu Gln Glu Ser Asp Cys Thr Glu Met Ser Trp Val Glu Ser Val
            195                 200                 205

CTT TAC TAC ACG GGC TTC CCC AGT GGT ACT CCA ACC ACG GCG CTC TTA        671
Leu Tyr Tyr Thr Gly Phe Pro Ser Gly Thr Pro Thr Thr Ala Leu Leu
        210                 215                 220

AGC CGT ACT CCT CAA AGA CTC AAC CCA TTC AAG                            704
Ser Arg Thr Pro Gln Arg Leu Asn Pro Phe Lys
    225                 230
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 234 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Val Asp Ile Glu Gln Glu Thr Ala Trp Val Gln Ala Gly Ala Thr Leu
  1               5                  10                  15

Gly Glu Val Tyr Tyr Arg Ile Ala Glu Lys Ser Asn Lys His Gly Phe
            20                  25                  30

Pro Ala Gly Val Cys Pro Thr Val Gly Val Gly Gly His Phe Ser Gly
        35                  40                  45

Gly Gly Tyr Gly Asn Leu Met Arg Lys Tyr Gly Leu Ser Val Asp Asn
    50                  55                  60

Ile Val Asp Ala Gln Ile Ile Asp Val Asn Gly Lys Leu Leu Asp Arg
65                  70                  75                  80

Lys Ser Met Gly Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly Gly
                85                  90                  95

Val Ser Phe Gly Val Val Leu Ala Tyr Lys Ile Lys Leu Val Arg Val
            100                 105                 110

Pro Glu Val Val Thr Val Phe Thr Ile Glu Arg Arg Glu Glu Gln Asn
        115                 120                 125

Leu Ser Thr Ile Ala Glu Arg Trp Val Gln Val Ala Asp Lys Leu Asp
    130                 135                 140

Arg Asp Leu Phe Leu Arg Met Thr Phe Ser Val Ile Asn Asp Thr Asn
```

```
145                 150                 155                 160

Gly Gly Lys Thr Val Arg Ala Ile Phe Pro Thr Leu Tyr Leu Gly Asn
                165                 170                 175

Ser Arg Asn Leu Val Thr Leu Leu Asn Lys Asp Phe Pro Glu Leu Gly
            180                 185                 190

Leu Gln Glu Ser Asp Cys Thr Glu Met Ser Trp Val Glu Ser Val Leu
        195                 200                 205

Tyr Tyr Thr Gly Phe Pro Ser Gly Thr Pro Thr Thr Ala Leu Leu Ser
    210                 215                 220

Arg Thr Pro Gln Arg Leu Asn Pro Phe Lys
225                 230
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1122 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1122

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
CAT CTT CTT TCA ACA TGT CAA ACT TCC ATT CTT ACT CTC CTT CTT CTC      48
His Leu Leu Ser Thr Cys Gln Thr Ser Ile Leu Thr Leu Leu Leu Leu
  1               5                  10                  15

TTG CTC TCA ACC CAA TCT TCT GCA ACT TCC CGT TCC ATT ACA GAT CGC      96
Leu Leu Ser Thr Gln Ser Ser Ala Thr Ser Arg Ser Ile Thr Asp Arg
            20                  25                  30

TTC ATT CAA TGT TTA CAC GAC CGG GCC GAC CCT TCA TTT CCG ATA ACC     144
Phe Ile Gln Cys Leu His Asp Arg Ala Asp Pro Ser Phe Pro Ile Thr
        35                  40                  45

GGA GAG GTT TAC ACT CCC GGA AAC TCA TCT TTT CCT ACC GTC TTG CAA     192
Gly Glu Val Tyr Thr Pro Gly Asn Ser Ser Phe Pro Thr Val Leu Gln
```

```
     50                  55                  60

AAC TAC ATC CGA AAC CTT CGG TTC AAT GAA ACT ACC ACA CCA AAA CCC      240
Asn Tyr Ile Arg Asn Leu Arg Phe Asn Glu Thr Thr Thr Pro Lys Pro
 65                  70                  75                  80

TTT TTA ATC ATC ACA GCC GAA CAT GTT TCC CAC ATT CAG GCA GCT GTG      288
Phe Leu Ile Ile Thr Ala Glu His Val Ser His Ile Gln Ala Ala Val
                 85                  90                  95

GTT TGT GGC AAA CAA AAC CGG TTG CTA CTG AAA ACC AGA AGC GGT GGT      336
Val Cys Gly Lys Gln Asn Arg Leu Leu Leu Lys Thr Arg Ser Gly Gly
            100                 105                 110

CAT GAT TAT GAA GGT CTT TCC TAC CTT ACA AAC ACA AAC CAA CCC TTC      384
His Asp Tyr Glu Gly Leu Ser Tyr Leu Thr Asn Thr Asn Gln Pro Phe
        115                 120                 125

TTC ATT GTG GAC ATG TTC AAT TTA AGG TCC ATA AAC GTA GAT ATC GAA      432
Phe Ile Val Asp Met Phe Asn Leu Arg Ser Ile Asn Val Asp Ile Glu
    130                 135                 140

CAA GAA ACC GCA TGG GTC CAA GCC GGT GCG ACT CTT GGT GAA GTG TAC      480
Gln Glu Thr Ala Trp Val Gln Ala Gly Ala Thr Leu Gly Glu Val Tyr
145                 150                 155                 160

TAT CGA ATA GCG GAG AAA AGT AAC AAG CAT GGT TTT CCG GCA GGG GTT      528
Tyr Arg Ile Ala Glu Lys Ser Asn Lys His Gly Phe Pro Ala Gly Val
                165                 170                 175

TGT CCA ACG GTT GGC GTT GGT GGG CAT TTT AGT GGT GGT GGG TAT GGT      576
Cys Pro Thr Val Gly Val Gly Gly His Phe Ser Gly Gly Gly Tyr Gly
            180                 185                 190

AAT TTG ATG AGA AAA TAT GGT TTG TCG GTT GAT AAT ATT GTT GAT GCT      624
Asn Leu Met Arg Lys Tyr Gly Leu Ser Val Asp Asn Ile Val Asp Ala
        195                 200                 205

CAA ATA ATA GAT GTG AAT GGC AAG CTT TTG GAT CGA AAG AGT ATG GGT      672
Gln Ile Ile Asp Val Asn Gly Lys Leu Leu Asp Arg Lys Ser Met Gly
    210                 215                 220

GAG GAT TTG TTT TGG GCG ATC ACC GGC GGT GGT GGT GTT AGT TTT GGT      720
Glu Asp Leu Phe Trp Ala Ile Thr Gly Gly Gly Gly Val Ser Phe Gly
225                 230                 235                 240

GTG GTT CTA GCC TAC AAA ATC AAA CTA GTT CGT GTT CCG GAG GTT GTG      768
Val Val Leu Ala Tyr Lys Ile Lys Leu Val Arg Val Pro Glu Val Val
                245                 250                 255

ACC GTG TTT ACC ATT GAA AGA AGA GAG GAA CAA AAC CTC AGC ACC ATC      816
Thr Val Phe Thr Ile Glu Arg Arg Glu Glu Gln Asn Leu Ser Thr Ile
            260                 265                 270
```

```
Phe Pro Ser Gly Thr Pro Thr Thr Ala Leu Leu Ser Arg Thr Pro Gln
        355                 360                 365

Arg Leu Asn Pro Phe Lys
  370
```

CLAIMS

1. An isolated protein obtainable from a plant source which has antifungal activity, preferably anti-Oomycete activity, more preferably anti-*Phytophthera* and/or anti-*Pythium* activity and a molecular weight of about 59 ± 5 kDa as judged by SDS PAGE-electrophoresis.

2. An isolated protein according to claim 1, characterised in that it is obtainable from sunflower plants.

3. An isolated protein, characterised in that it comprises one or more of the peptides selected from the group consisting of:
(a) amino acids 1 to 25 of SEQIDNO: 1,
(b) amino acids 1 to 25 of SEQIDNO: 2,
(c) amino acids 1 to 118 of SEQIDNO: 6,
(d) amino acids 1 to 132 of SEQIDNO: 11,
(e) amino acids 1 to 234 of SEQIDNO: 13,
(f) amino acids 1 to 374 of SEQIDNO: 15,
as well as muteins thereof which have antifungal activity.

4. An antifungal protein comprising an amino acid sequence characterised in that it is capable of being encoded by the open reading frame represented by SEQIDNO: 10, or by part of said open reading frame.

5. An antifungal protein comprising an amino acid sequence characterised in that it is capable of being encoded by the open reading frame represented by SEQIDNO: 12, or by part of said open reading frame.

6. An antifungal protein comprising an amino acid sequence characterised in that it is capable of being encoded by the open reading frame represented by SEQIDNO: 14, or by part of said open reading frame.

7. An isolated DNA sequence comprising an open reading frame capable of encoding a protein according to any of the claims 1 to 6, and DNA capable of hybridising therewith under stringent conditions.

8. An isolated DNA sequence according to claim 7, characterised in that it comprises the nucleotide sequence depicted in SEQIDNO: 10.

9. An isolated DNA sequence according to claim 7, characterised in that it comprises the nucleotide sequence depicted in SEQIDNO: 12.

11. An isolated DNA sequence according to claim 7, characterised in that it comprises the nucleotide sequence depicted in SEQIDNO: 14.

12. A chimeric DNA sequence comprising a DNA sequence according to any of claims 7 to 12.

13. A chimeric DNA sequence according to claim 12, further comprising a transcriptional initiation region and, optionally, a transcriptional termination region, so linked to said open reading frame as to enable the chimeric DNA to be transcribed in a living host cell when present therein, thereby producing RNA which comprises said open reading frame.

14. A chimeric DNA sequence according to claim 13, wherein the RNA comprising said open reading frame is capable of being translated into protein in said host cell, when present therein, thereby producing said protein.

15. A chimeric DNA sequence according to any one of claims 12 to 14 which is a replicon, preferably pMOG1144.

16. A chimeric DNA sequence according to claim 15 which is a vector.

17. A vector according to claim 16, which is a binary vector, preferably pMOG1144.

18. A host cell comprising a replicon according to claim 15 and which is capable of maintaining said replicon once present therein.

19. A host cell comprising a vector according to claim 16 or 17 and which is capable of maintaining said vector once present therein.

20. A host cell stably incorporating in its genome a chimeric DNA sequence according to claim 12 or 13.

21. A host cell according to claim 19 which is a plant cell, said vector being a non-integrative viral vector.

22. A host cell according to claim 20 which is a plant cell.

23. A plant or a plant part comprising at least one plant cell according to claim 21 or 22.

24. A plant or a plant part consisting essentially of plant cells according to claim 22.

25. A plant according to claim 24, characterised in that said chimeric DNA is expressed in at least a number of the plant's cells causing the said antifungal protein to be produced therein.

26. A method for the production of a protein with antifungal activity, preferably anti-anti-Oomycete activity, more preferably anti-*Phytophthora* activity and/or anti-*Pythium* activity, characterised in that a host cell according to claim 19 or 20 is grown under conditions allowing the said protein to be produced by said host cells.

27. A method according to claim 26, further comprising the step of recovering the protein from the host cells.

28. Use of a protein according to any one of claims 1 to 6 for retarding fungal growth, preferably Oomycete growth and more preferably the growth of *Phytophthora sp.* and/or *Pythium sp.*

29. The use according to claim 28, characterised in that spores of the said fungus are caused to be contacted with said protein.

30. A method of retarding the growth of the a fungus, preferably an Oomycete, more preferably *Phytophthora* or *Pythium* on plant leaves, characterised in that hyphae thereof, or spores thereof, are caused to be contacted with a protein produced from a host cell according to claim 19 or 20, or from a cell of a plant according to claim 25.

31. A method for obtaining plants with reduced susceptibility to fungi, preferbly Oomycetes, more preferably *Phytophthora* or *Pythium*, comprising the steps of
(a) introducing into ancestor cells which are susceptible of regeneration into a whole plant,
- a chimeric DNA sequence comprising an open reading frame capable of encoding a protein according to any of claims 1-6, said open reading frame being operatively linked to a transcriptional and translational region and, optionally, a transcriptional termination region, allowing the said protein to be produced in a plant cell that is susceptible to infection by said fungus and
- a chimeric DNA sequence capable of encoding a plant selectable marker allowing selection of transformed ancestor cells when said selectable marker is present therein, and

(b) regenerating said ancestor cells into a plant under conditions favouring ancestor cells which have the said selectable marker, and
(c) identifying a plant which produces a protein according to claim 1, thereby reducing the susceptibility of said plant to infection by said fungus.

32. The method according to claim 31, characterised in that step (a) is performed using an

Agrobacterium tumefaciens strain capable of T-DNA transfer to plant cells and which harbours a binary vector, and wherein step (b) is performed in the presence of an antibiotic favouring cells which have a neomycin phosphotransferase.

33. An antifungal composition comprising a protein according to any one of claims 1 to 6, and a suitable carrier.

34. An antibody capable of recognising a protein according to any one of claims 1 to 6.

35. A nucleic acid sequence obtainable from a gene encoding a protein according to any one of claims 1 to 6, having tissue-specific and/or developmental specific transcriptional regulatory activity in a plant.

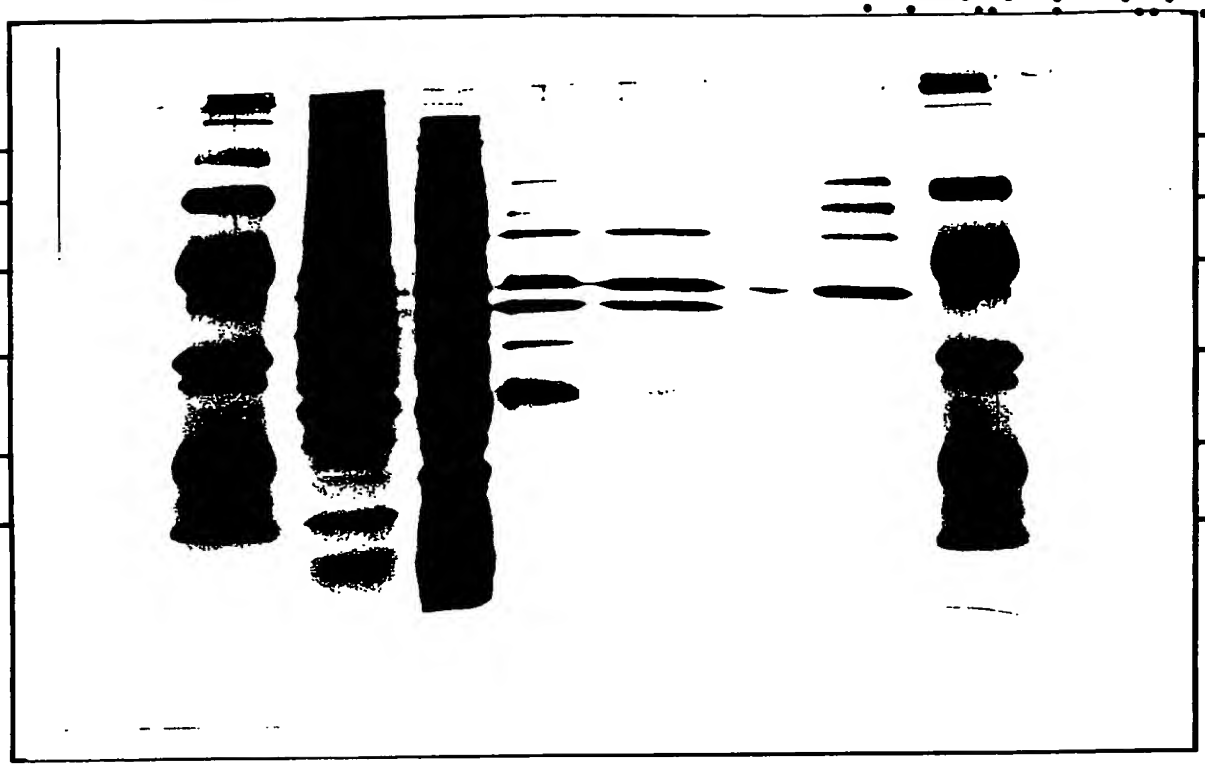
36. A nucleic acid sequence according to claim 35, which is obtainable from the region upstream of the translational initiation site of said gene.

37. A nucleic acid sequence according to claim 36, which has at least 1000 nucleotides of said region upstream of the translational initiation site of said gene.

38. Use of a nucleic acid sequence according to any one of claims 35 to 37 for making a plant expressible gene construct.

ABSTRACT

The present invention provides an isolated protein obtainable from a plant source which has antifungal activity, specifically anti-*Phytophthora* activity and/or anti-*Pythium* activity and a molecular weight of about 59 ± 5 kDa as judged by SDS PAGE-electrophoresis, an isolated DNA sequence comprising an open reading frame capable of encoding a protein according to the invention, preferably characterised in that it comprises an open reading frame which is capable of encoding a protein as represented by amino acids 1 to 374 of SEQIDNO: 15 or muteins thereof, and DNA capable of hybridising therewith under stringent conditions. The invention further comprises plants incorporating chimeric DNA capable of encoding a protein according to the invention, and wherein the protein is expressed. Also methods are provided for combating fungi, especially *Phytophthora* and *Pythium* species, using a protein or a host cell capable of producing the protein.



Figure

Figure 2 2/3

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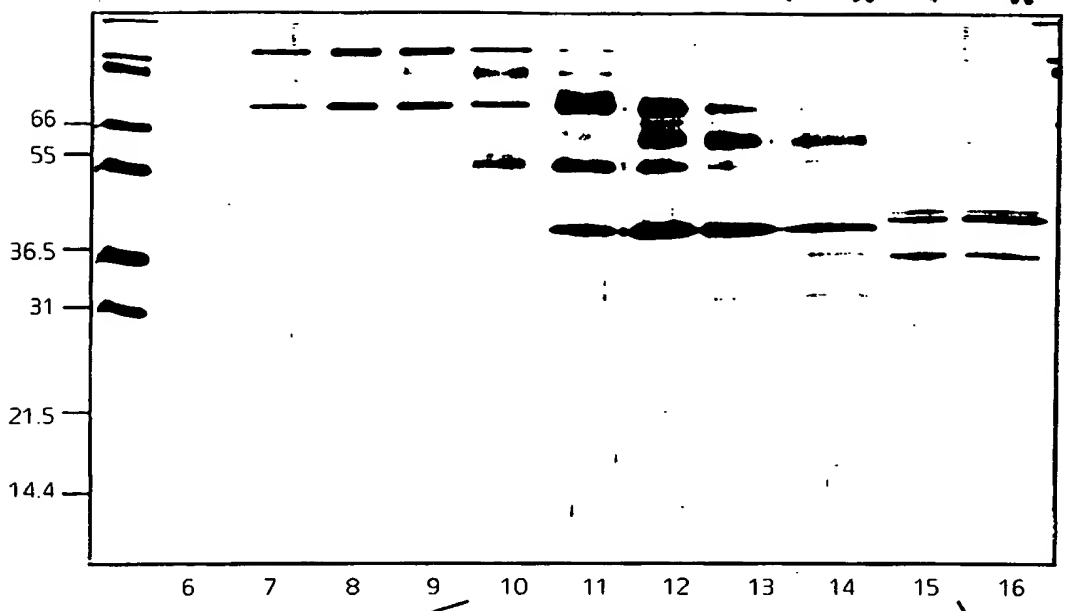

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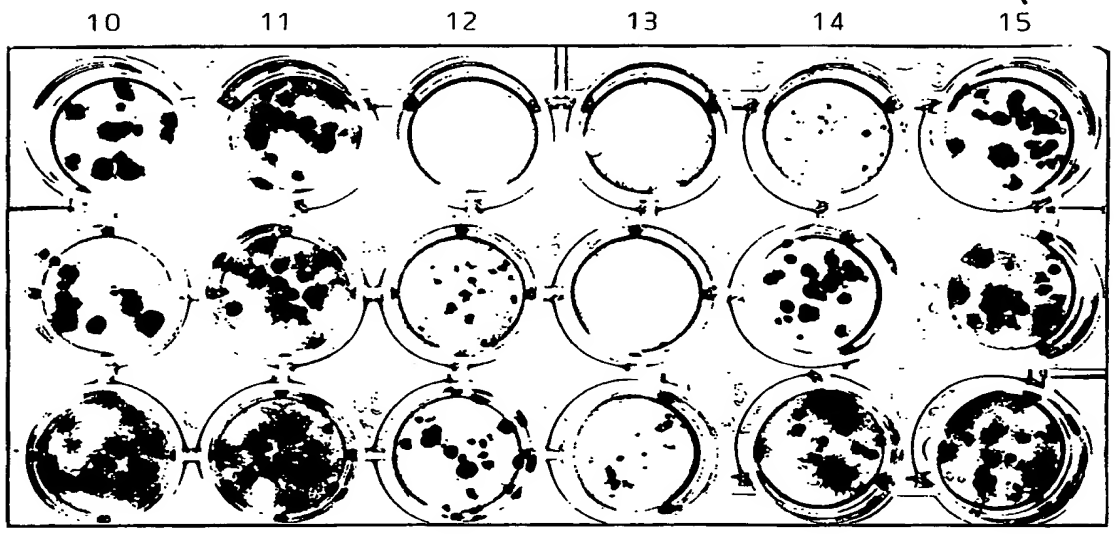

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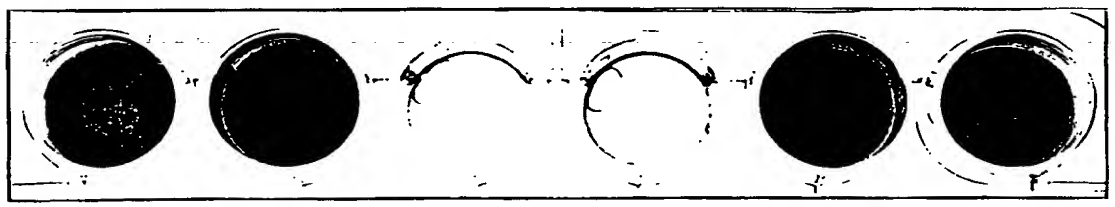

Figure 3

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